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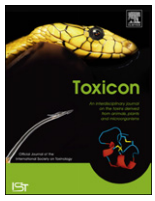

Short communication

Preclinical testing of Peruvian anti-bothropic anti-venom against *Bothrops andianus* snake venom

Francisco S. Schneider [a], Maria C. Starling [a], Clara G. Duarte [a], Ricardo Machado de Avila [a], Evanguedes Kalapothakis [a], Walter Silva Suarez [b], Benigno Tintaya [b], Karin Flores Garrido [b], Silvia Seraylan Ormachea [b], Armando Yarleque [c], César Bonilla [b], Carlos Chávez-Olórtegui [a,*]

[a] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CP: 486, CEP: 31270-901, Belo Horizonte, Minas Gerais, Brazil*
[b] *Instituto Nacional de Salud, Lima, Peru*
[c] *Universidad Nacional Mayor de San Marcos, Lima, Peru*

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ABSTRACT

Bothrops andianus is a venomous snake found in the area of Machu Picchu (Peru). Its venom is not included in the antigenic pool used for production of the Peruvian anti-bothropic anti-venom. *B. andianus* venom can elicit many biological effects such as hemorrhage, hemolysis, proteolytic activity and lethality. The Peruvian anti-bothropic anti-venom displays consistent cross-reactivity with *B. andianus* venom, by ELISA and Western Blotting and is also effective in neutralizing the venom's toxic activities.

Snake bites represent an important health problem in Peru, especially to the east of the Andes in the High Forest (600–3500 m altitude) and Tropical Rain Forest (<600 m altitude) (Ministério de Salúd Peru, 2004). These regions are known for containing the major Peruvian snake species and most diversified ophidian population. The Instituto Nacional de Salud (INS), located in Lima, Peru has been producing commercial anti-venoms since 1978 (Ministério de Salúd Peru, 2004). The Peruvian anti-bothropic polyvalent anti-venom (PABA), used to treat envenomations involving Bothrops complex (*Bothrops* and *Bothrocophias* genera), is a whole IgG preparation produced from horse plasma previously immunized with an antigenic pool consisting of *Bothrops atrox* (50%), *Bothrops pictus*, *Bothrops barnetti*, *Bothrops brazili* and *Bothrocophias hyoprora* (12.5% each) venoms (Laing et al., 2004; Rojas et al., 2005; Theakston and Warrell, 1991). Besides neutralizing the most severe toxic effects induced by envenomation involving snakes from the antigenic pool, (Laing et al., 2004; Rojas et al., 2005) the preclinical assessment of anti-venom's efficacy against venoms from other medically important species would be useful in Latin America for improving anti-venom production (Gutierrez et al., 2009). This work describes the preclinical evaluation of the neutralizing capacity of PABA against lethality, hemorrhagic, proteolytic, and PLA_2 effects of *Bothrops andianus*' venom. *B. andianus* is a venomous snake found in the southern mountains of Peru and Bolivia and its venom is

Abbreviations: INS, Instituto Nacional de Salud; PABA, Peruvian anti-bothropic polyvalent anti-venom; BSA, Bovine serum albumin; PBS, Phosphate buffered saline; LD_{50}, Median lethal dose; MHD, Minimum hemorrhagic dose; MPD, Minimum PLA_2 Dose; DMC, Dimethylcasein; ED_{50}, Median effective dose.

* Corresponding author. Tel.: +55 31 3409 2625; fax: +55 31 3441 5963.
E-mail address: olortegi@icb.ufmg.br (C. Chávez-Olórtegui).

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not included in PABA production. In Peru, *B. andianus* is found in the areas (departments) of Cuzco and Puno, at elevations of 1800–3300 m (Ministério de Salúd Peru, 2004). Its geographical distribution overlaps Machu Picchu area, a UNESCO World Heritage Site (UNESCO, 2012), which is an important touristic attraction and receives more than 600,000 tourists per year, increasing the risks of accidents involving this snake. In Peru, the snakes of genus *Bothrops* are responsible for 80% of accidents and approximately 6.5% of these accidents are registered in the Cuzco and Puno Departments (Ministério de Salúd Peru, 2004).

For the experiments, male and female Swiss mice (18–22 g) were maintained in the Centro de Bioterismo of Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais (UFMG), Brazil. All animals received water and food *ad libitum* under controlled environmental conditions. The experimental protocols were approved by the Ethics Committee in Animal Experimentation (CETEA/UFMG). PABA, crude venoms from *B. andianus*, and antigenic pool species were provided by INS. Venoms were kept at −20 °C and anti-venom at 4 °C temperature as indicated on their prescription. The protein content in crude venoms and anti-venoms were determined according to Bradford's method (1976) using BSA (Sigma Chemicals) as standard.

Lethality of *B. andianus* venom was assessed by the intra-peritoneal (i.p.) route. Groups of four mice were injected with increasing amounts of venom (34.6 μg–72 μg/mouse), dissolved in 0.5 ml of PBS–BSA 0.01% solution, pH 7.4. Twenty four hours later, deaths were counted and LD_{50} was calculated using Probit analysis (95% confidence) (Finney, 1971).

The hemorrhagic activity was assayed as described in Kondo et al. (1960) and modified by Gutierrez et al. (1985). Five different doses (3.72 μg; 5.2 μg; 7.29 μg; 10.2 μg; 14.28 μg) of crude venom were inoculated subcutaneously into dorsal shaved skin of mice in 0.1 ml NaCl 0.9%. Two hours later, mice were sacrificed by cervical dislocation and back skin was totally removed in order to measure the area of the hemorrhagic lesion. MHD was defined by the dose causing a lesion with a diameter of 10 mm.

PLA_2 activity was measured using an indirect hemolytic assay (Gutierrez et al., 1988). Increasing concentrations of *B. andianus* venom (from 0.004 μg up to 10 μg) were prepared in a final volume of 15 μl in PBS and added to 2 mm wells in agarose gel plates (0.8% in PBS, pH = 8.1, containing 1.2% sheep erythrocytes, 1.2% egg yolk and 100 mM $CaCl_2$). Plates were incubated at 37 °C for 18 h and the diameters of the hemolytic haloes were measured. In controls, 15 μl of PABA was used. One unit (Minimum PLA_2 Doses-MPD) corresponds to a minor concentration of venom which produced a hemolytic halo of 10 mm diameter. Experiments were conducted in triplicate.

Proteolytic activity was measured with dimethylcasein (Sigma) as described in Lin et al. (1969) with the modifications described in Sanchez et al. (2000). Dilutions corresponding to 5, 10, 20 and 40 μg of venom were used and absorbance values were determined at 340 nm. One unit was defined as ΔA 340 nm/min. Activity was expressed relative to protein concentration (mg).

The anti-venom potency was determined by mixing $5LD_{50}$ of *B. andianus* venom with 12.5, 25, 50, 100 or 200 μl of PABA and incubating for 1 h at 37 °C followed by i.p. injection in 5 groups of 4 mice. Median effective dose (ED_{50}) was calculated from the number of deaths within 24 h of injection of the venom/anti-venom mixture using Probit analysis as described above. The ED_{50} was expressed as ml anti-venom/mg of venom needed to prevent death in 50% of the injected mice.

To determine the neutralization of hemorrhagic activity, PABA was incubated with either 3MHD or 5MHD for 30 min at 37 °C according to manufacturer's instructions (1 μL of serum to 2.5 μg of venom) and inoculated in different groups of 3 Swiss male mice (18–22 g) as described above. Positive and negative control groups, each consisting of 2 mice were treated with venom alone (5MHD) or anti-venom, respectively. Two hours later, mice were euthanized and the hemorrhage was measured (Kondo et al., 1960; Sanchez et al., 1992).

Inhibition of PLA_2 activity of *B. andianus* venom by PABA was conducted as described by Gutierrez et al. (1998). Two MPD of venom were incubated with 13, 6.5 and 3.25 μl of anti-venom for 30 min at 37 °C, and 15 μl of each mixture added in triplicate to wells in agarose gels. Neutralization of venom was checked by the absence of halos on the plate's surface.

Inhibition of dimethylcasein hydrolysis by PABA was estimated by incubation (30 min at 37 °C) of a fixed concentration of *B. andianus* venom with increasing amounts of anti-venom (μl). After incubation the mixtures were tested as described before. The neutralizing ability of anti-venom is expressed as the quantity of anti-venom able to neutralize 50% of proteolytic activity obtained by 1 mg of venom.

For the immunological assays (ELISA and Western Blotting assays), Falcon flexible micro titration plates were used (Becton Dickinson France S.A). The plates were coated overnight at 5 °C with 100 μl of a 5 μg/ml solution of the crude venoms (*B. andianus*, *B. atrox*, *B. barnetti*, *B. brazili*, *B. pictus* and *B. hyoprora*) in 0.02 M sodium bicarbonate buffer, pH 9.6. The assays were performed as described previously by Chávez-Olórtegui et al. (1991). Absorbance values were determined at 492 nm with a Biorad 680 Microplate Reader. All measurements were made in triplicate and the results expressed as the median of two assays. For Western Blotting the venoms were subjected to electrophoresis SDS-PAGE (15%) according to Laemmli (1970) in reducing conditions. The proteins were transferred onto nitrocellulose membranes (Towbin et al., 1979) and blocked with PBS-Tween 0.3% containing 2% casein. The membranes were incubated with PABA (1:10,000) for 1 h at room temperature. Immunoreactive proteins were detected using anti-horse Sigma IgG conjugated with peroxidase (1:3000). After washing three times for 5 min with PBS-Tween 0.05%, blots were developed using DAB/chloronaphthol according to the manufacturer's instructions.

The LD_{50} of *B. andianus* venom determined in this paper (57.96 μg, Table 1) is similar to the LD_{50} doses of *B. atrox*, 49.9 μg/mouse; *B. pictus*, 58.91 μg/mouse and *B. Barnetti*, 53.2 μg/mouse (Laing et al., 2004; Rojas et al., 2005). However, *B. brazili* venom was three times more potent in the LD_{50} assay than the other four Peruvian venoms (Laing et al., 2004). PABA was effective in neutralizing lethality

Table 1
Biological activities of *Bothrops andianus* venom and their neutralization by PABA.

Effect	Activity	Neutralization[e] (μl anti-venom/mg venom)
Lethal[a]	LD_{50} = 57.96 μg (36.02–93.26)	200 ± 0.0
Hemorrhagic[b]	MHD = 4.68 μg ± 0.20	187.8 ± 6.0
Proteolytic[c]	68.50 U/mg ± 1.75	500.0 ± 43.0
PLA_2 activity[d]	MPD = 5 μg ± 2.83	350 ± 40.0

Results are presented as mean ± S.D. (n = 4), except in lethality, where 95% confidence limits are included in parentheses.

[a] LD_{50} is expressed as the dose of venom (μg) responsible for causing 50% of death in mice (18–22 g) i.p. injected.

[b] MHD is defined as the amount of venom (μg) causing a 10 mm diameter spot hemorrhage on skin 2 h after injection.

[c] Proteolytic activity is expressed as Units/mg of venom. One unit of specific activity is defined as ΔA 340 nm/min.

[d] Minimum PLA_2 Dose (MPD) is defined as the amount of venom responsible for causing a 10 mm diameter of hemolysis.

[e] Neutralization corresponds to the quantity of anti-venom able to neutralize in 50% lethal, hemorrhagic, proteolytic and PLA_2 activities obtained by 1 mg of venom.

induced by *B. andianus* venom and showed high neutralizing potency (Table 1, ED_{50} of 200 μl anti-venom/mg venom). Furthermore, local hemorrhagic activity of *B. andianus* venom was evaluated in a mouse model. *B. andianus* venom directly induced extra vascular bleeding on the underside of the skin 2 h after injection. The estimated MHD is 4.68 μg ± 0.20 (Table 1). The results obtained concerning the capacity of PABA to neutralize the hemorrhagic effect of *B. andianus* are shown in Table 1. This antivenom was efficient in neutralizing the hemorrhagic activity. MPD using an indirect hemolytic assay and inhibition of PLA_2 activity by PABA were measured. PLA_2 activity was dose dependent (data not shown) and the MPD determined in this study was 5.0 μg (S.D. ± 2.83 μg) (Table 1). PABA was also able to neutralize *B. andianus* PLA_2 activity with a potency of 350 ± 40.0 (μl anti-venom/mg venom). The proteolytic activity of *B. andianus* venom was expressed as DMC units (Δ340 nm) hydrolyzed per mg of venom per minute and was found to be 68.5 U/mg min ± 1.75 (Table 1). PABA was able to neutralize *B. andianus* proteolytic activity with a potency of 200 ± 11.4 (μl anti-venom/mg venom).

Immunological cross-reactivity of PABA against *Bothrops* venoms was assessed by both ELISA and western blotting. For ELISAs, reactivity at different serum dilutions can be seen in Fig. 1A. Importantly, cross-reactivity with *B. andianus* venom and reactivity with *B. atrox*, *B. barnetti* and *B. pictus* was observed. In this experiment, a weaker reactivity was observed against the venoms from *B. pictus* and *B. hyoprora*. Fig. 1B shows the results of the Western Blot assay. PABA was able to recognize all of the analyzed venoms. Regarding *B. andianus* venom, reactivity against bands at ~14, 25, 50 kDa and higher masses were observed. There was remarkable reactivity with the ~14 kDa protein compared to the others.

B. andianus venom has toxicological and electrophoretic profiles similar to those of other Peruvian *Bothrops* sp. venoms used in the anti-venom production. The

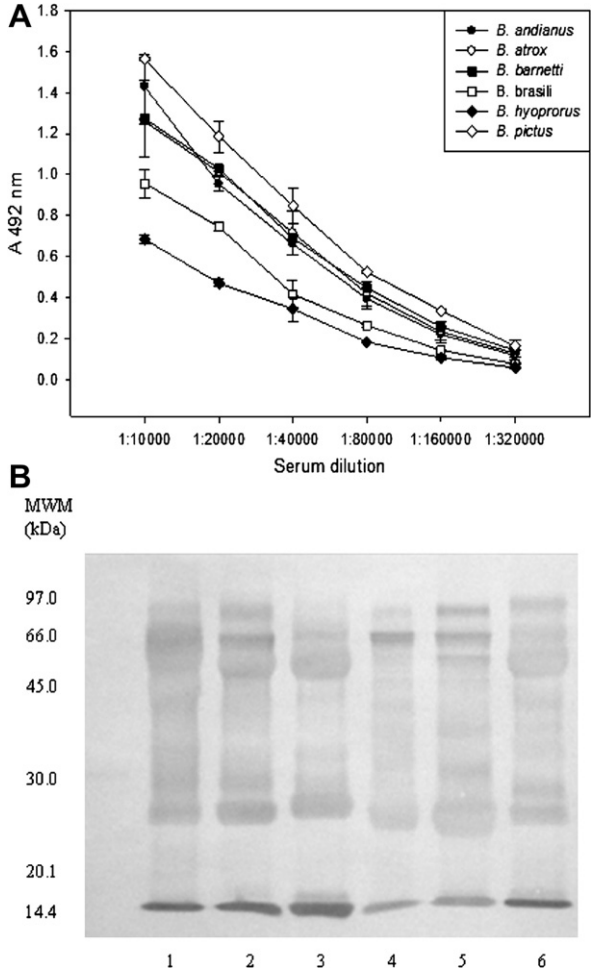

Fig. 1. (A) ELISA reactivity of Peruvian anti-bothropic serum against *Bopthrops andianus*, *B. atrox*, *B. barnetti*, *B. brazili*, *B. hyoprora* and *B. pictus* venoms. Plates were coated with 0.5 μg of each venom per well and cross-reactivity tested against anti-bothropic anti-venom using serial dilution from 1:2000 to 1:256,000. Antibodies were detected with anti-horse Sigma IgG conjugated with peroxidase (1:3000), OPD and H_2O_2 in citrated buffer. (B) Western Blotting analysis of *Bothrops* venoms. 20 μg of each of the venoms were analyzed by SDS-PAGE under reducing conditions. Lanes correspond to: (1) *B. andianus*, (2) *B. atrox*, (3) *B. barnetti*, (4) *B. brazili*, (5) *Bothrocophias hyoprora*, (6) *B. pictus*. The venoms were analyzed against anti-bothropic anti-venom (1:10,000) and revealed with DAB/chloronaphthol.

toxicological profile is also common to Bothropic envenomations characterized by local tissue damage and by systemic manifestations (White, 2005). The symptoms observed in animals experimentally envenomed by *B. andianus* venom were very similar to other Peruvian *Bothrops* venoms (Laing et al., 2004; Rojas et al., 2005). Our observations find that PABA is effective in neutralizing the most important toxic activities induced by *B. andianus* venoms when using an experimental protocol based on pre-incubation of venom and anti-venom before testing in experimental systems (Gutierrez et al., 1990; Otero et al., 1995). Thus, despite the fact that *B. andianus* venom is not included in the antigenic pool used in Peru, PABA is

effective against this venom. Our preclinical observations are in agreement with the report of Rojas et al. (2005), which shows the efficacy of Peruvian anti-venom in neutralizing many snake venoms found in Peru.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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